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1. **Lam et al. Life Sciences, 1998, 62(17-18), pp. 1577-1583.**
2. **Lou et al. Bioorg. Med. Chem. May 1996, 4(5), pp. 677-682.**

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APPLICATION OF "ONE-BEAD ONE-COMPOUND" COMBINATORIAL LIBRARY METHODS IN SIGNAL TRANSDUCTION RESEARCH

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Summary

Using a "split-synthesis" solid phase synthetic approach, bead libraries can be generated such that each bead displays only one chemical entity. This "one-bead one-compound" combinatorial library can then be assayed for specific biological properties using either a solid-phase on-bead binding or functional assay, or a releasable solution phase assay. Positive compound-beads can then be isolated for structure determination. Various assay systems to screen such a "one-bead one-compound" library are described. We have used this combinatorial library method to discover peptides that bind to the cell surface immunoglobulins of murine lymphoma cells. Such peptides, when presented in an oligomeric form to a lymphoma cell are able to induce signal transduction. Additionally, we have also applied the "one-bead one-compound" combinatory library approach to elucidate peptide substrate motifs for protein tyrosine kinases. Multiple distinct peptide motifs were identified for $p60^{c\text{-}src}$ protein tyrosine kinase. Using the identified peptide substrates as templates, potent and highly specific pseudosubstrate-based peptide inhibitors were developed.

Key Words: **combinatorial chemistry, synthesis peptide libraries, signal transduction, protein kinase**

The field of combinatorial chemistry has exploded in the last six to seven years. Many reviews on the subject have been written (1). Although combinatorial chemistry was first developed by peptide chemists (2,3,4,5), the techniques have now been applied by many medicinal chemists and organic chemists for the development of small molecule drugs (6,7). Combinatorial library methods involve (i) the production of a large number of compounds by

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randomly joining various components (or building blocks) together, (ii) a high throughput assay method for a specific biological, chemical, or physical property, and (iii) structure determination of the active compound by a deconvolution method, by direct chemical analysis, or by analysis of the coding structure. There are four general approaches to synthetic combinatorial library methods: (i) spatially addressable parallel solid phase or solution phase libraries (2,8,9), (ii) synthetic library methods requiring deconvolution (3,10,11,12,13), (iii) "one-bead one-compound" library method (4,14,15,16), and (iv) synthetic library method using affinity chromatography selection (17,18,19). Chemical libraries containing peptides, non-peptide oligomers, or small molecules can be generated by synthetic methods and screened with the above approaches (6,7,14). More recently, methods have been developed to use enzymes or whole organisms to generate organic molecules that are otherwise very difficult to synthesize (20,21). This biocatalytic synthetic approach are likely to have important impact in the field of combinatorial chemistry in the future.

We first described the "one-bead one-compound" combinatorial library method in 1991 (4). Since then, this approach has been applied by us and others (14) to develop ligands for many macromolecular targets. In this method, the chemical library was first prepared by a "split-synthesis" approach (3,4,22) on solid-phase. As a result, each bead will display only one chemical species but there are approximately 10^{13} copies of the same chemical compound on and within one single bead (100 μm diameter). Using an appropriate detection scheme, individual compound-bead of a specific biological, biochemical, or physical property can be isolated for subsequent structure determination. A comprehensive review on this library method has been published recently (14). In this paper, we report on our experience of using the "one-bead one-compound" combinatorial library method for signal transduction research.

Methods

Preparation of "one-bead one-compound" combinatorial libraries

The "one-bead one-compound" combinatorial libraries are prepared by a "split-synthesis" approach (3,4,22). Many commercially available resins can be used (14). We commonly use the amino-polyethylene glycol grafted polystyrene beads (or Tenta Gel-S, Rapp Polymere, Germany) as the solid-phase support. In the case of peptide libraries, the resins are first split into multiple equal aliquots, after which 4 fold excess of individual Fmoc-amino acids, together with the coupling agents (e.g. HOBt and BOP) are added. After the coupling reactions are completed, the resins are mixed together, thoroughly washed, and the N-α-Fmoc group deprotected with 20% piperidine (v/v). The beads are then washed thoroughly, split into several aliquots again, and made ready for the next cycle of coupling. After several coupling cycles, the N-α-Fmoc and side chain protecting groups are removed with piperidine and trifluoroacetic acid (TFA) respectively. Since the bead does not have any cleavable linkers, the deprotected peptides remain attached to the bead via a polyethylene glycol linker. In some applications, it may be desirable for the peptide to be released from the bead so that the peptide can be assayed in solution phase. In this case, resin with an appropriate cleavable linker can be used (14).

TentaGel is compatible with many organic reactions and solvents and therefore may also be used in the preparation of synthetic small molecule libraries. Solid-phase synthetic peptide chemistry has been very well-developed since its invention by Bruce Merrifield in 1963 (23).

The coupling rate is in excess of 95% in most cases, therefore, the quality of the peptide bead-library is usually very good. In contrast, solid-phase synthetic organic chemistry is not as well developed and the coupling efficiency of many organic reactions are far from 90%. Currently, efforts have been directed to improve the coupling efficiency of many organic reactions on solid phase (6,7,14).

Screening of "one-bead one-compound" combinatorial libraries

A reliable high-throughput assay method is needed for the evaluation of combinatorial libraries. The rate limiting step of the "one-bead one-compound" combinatorial library is often the final step of structure determination of the active compound-bead. From our experience, it is extremely important to include steps in the screening procedure so that most of the false positive beads can be eliminated prior to structure determination. Both on-bead binding or on-bead functional assays as well as releasable solution phase assays can be applied to the bead-library. We have in the last few years applied the on-bead binding and functional assays for various areas of signal transduction research (14). Others have used the releasable solution phase assay for identification of antagonists and agonists for G-protein coupled receptors (24). Recently, we described the use of an *in situ* releasable solution phase assay for the discovery of anti-cancer agents (25).

In the on-bead binding assays, the bead-library is first mixed with the target. The target could be a protein such as an antibody or a cellular receptor. It could also be a whole cell. The protein target needs to be tagged with a reporter group, such as an enzyme (e.g. alkaline phosphatase), a fluorescent molecule, or a radionuclide (14). The enzyme-linked colorimetric assay is the most convenient and efficient approach for screening a bead-library. One to ten million beads can usually be screened by a single researcher in one to two working days. Briefly, the enzyme-linked receptor is incubated with the bead-library for a few hours, after which the bead-library is thoroughly washed and a substrate such as 5-bromo-4-chloro-3-indoyl phosphate (BCIP) is added. The positive beads will turn turquoise and can easily be isolated over a light box and then under a dissecting microscope with a hand-held micropipette. The positive beads are then decolorized with dimethylformamide, recycled and subjected to the same screening procedure as described above except that this time, excess known ligand is added. In this way, the true-positive beads will not be stained in the second round and therefore can be isolated for microsequencing.

Recently, we described a whole-cell binding assay to discover peptides that bind to the surface of intact cells (26). In this method, the bead library is first mixed with intact cells for approximately one hour after which most of the free cells are removed and the remaining bead library examined under a dissecting microscope. A monolayer of cells is found to attach to the surface of some of the beads. These positive beads are then isolated, treated with 8M guanidine hydrochloride, pH 1.0, and recycled for a second round of cell binding study but in the presence of a blocking monoclonal antibody that binds to a specific receptor. Inhibition of cell binding to a bead by such an antibody in a second step suggests that the ligand on the bead interacts with that specific receptor. More recently, we developed a multi-color whole-cell binding assay in which different cell types are fluorescently labelled with a different color (i.e. different emission wavelengths). With an appropriate filter set, beads that bind to one but not the other cell types can be identified under the fluorescent microscope in one single step (Lam et al., unpublished data).

In the last few years, we have applied the "one-bead one-compound" combinatorial library method to identify peptide substrate motifs for protein tyrosine kinases (27,28,29). In this on-bead functional assay, we first mix the peptide bead library with [γ^{32}P]-ATP and a specific protein kinase. After incubation for an hour, the beads are treated with HCl to hydrolyze the ATP (to eliminate non-specific binding). After thorough washing, the beads are immobilized on a glass plate with agarose. [^{32}P]-labelled beads are then localized by autoradiography and subsequently physically isolated under a dissecting microscope. Because the beads are densely plated in the first round of screening, negative beads in close proximity to the positive beads are also isolated. In order to isolate individual positive beads, we melt the agar containing the positive beads, dilute and immobilize them with excess agarose prior to exposure to X-ray film. In this way, the positive beads are far apart from the negative beads, and therefore can be individually localized and isolated without difficulty.

Results

Identification of Idiotype-specific Peptides for B-cell Lymphoma

Using an enzyme-linked colorimetric assay with purified surface idiotypes from murine lymphoma lines (WEHI-231 and WEHI-279) as probes, we succeeded in identifying a series of all L-amino acid and all D-amino acid containing peptides that bind specifically to the surface idiotype of the lymphoma cells (30). When one of the all D-amino acid idiotype-specific peptides (against WEHI 231 murine lymphoma), wGeyidk, was biotinylated and tetramerized by mixing with avidin, this peptide induced signal transduction in intact cells as shown by an increase in tyrosine phosphorylation. More recently, we assembled the same peptide on an octameric aminopolyethylene glycol scaffold (M.W. 25,000) and showed that this peptide oligomer was capable of triggering tyrosine phosphorylation at a concentration of 10 nM (unpublished data). Preliminary studies showed that when biotin-wGeyidk/bodipy-avidin complex was mixed with intact WEHI-231 cells, the fluorescent label began to appear in the cytoplasm in 5 minutes. After 30 minutes, some fluorescent label was detected in the nucleus and by 2 hours, large amounts of fluorescent label migrated to the nucleus.

We proposed that idiotype-specific peptides (31) or other cell surface binding peptides (32) can be used as targeting agents for B-cell lymphoma. Currently, work is underway in our laboratory in using the multi-color whole cell binding approach to identify peptides that are specific against B-cell antigens (such as CD19, 20, 22), since monoclonal antibodies against these pan-B specific antigens have been proven clinically useful for the treatment of human lymphoma (33).

Identification of Integrin-specific Peptides for Human Prostate Cancer

Using the whole cell binding assay and blocking with anti-$\alpha_6\beta_4$-integrin antibody in the second step of screening, we identified two peptides that bind to a human prostate cancer cell line (DU145) (26). These two peptides, LVIVSVNGRHX (RU-1) and DNRIRLQAKXX (RX-2), resemble the previously reported active peptide sequences (GD-2 and AG-73) from native laminin. These peptides, in solution form, bind to DU145 cells as shown by FACS analysis. They also support cellular binding when immobilized on the 96-well plate.

Identification of Peptide Substrates and Development of Inhibitors for Protein Kinase

Using the on-bead functional assay described above, we have identified peptide substrate motif for cAMP dependent protein kinase (27), $p60^{c\text{-}src}$ protein tyrosine kinase (PTK) (28,29), and c-abl PTK. By screening a completely random heptapeptide library XXXXXXX (wherein X = 19 eukaryotic amino acids except Cys), YIYGSFK was initially identified as an efficient (Km = 55 μM) and specific substrate for $p60^{c\text{-}src}$ PTK (28). Using YIYGSFK as a template, and replacing Tyr^3 with various tyrosine analogs, we determined that YI(2'-Nal)GSFK was a relatively potent pseudosubstrate inhibitor for $p60^{c\text{-}src}$ PTK (29). Extensive structure-activity relationship study of YIYGSFK led us to conclude that -Ile-Tyr- were the two critical residues and that Tyr^3 was the phosphorylation site (34). Based on this dipeptide motif, a secondary library was synthesized (XIYXXXX). From this secondary library, GIYWHHY was found to be a more efficient substrate (Km = 20 μM) than the parent compound YIYGSFK (29). Using GIYWHHY as a template, we designed and synthesized several potent pseudosubstrate inhibitors for $p60^{c\text{-}src}$ PTK. Three of these inhibitors GI(2'-Nal)WHH(2'-nal), GI(2'-nal)WHH(2'-nal), and GI(2'-Nal)WHH(2'-Nal) have an IC_{50} of approximately 4 μM (35). Kinetics analysis (Lineweaver-Bark plots) of the inhibition of the $p60^{c\text{-}src}$ PTK activity by GI(2'-Nal)WHHY or GI(2'-Nal)WHH at various concentration of YIYGSFK substrate demonstrated noncompetitive inhibition suggesting that GIYWHHY and YIYGSFK may bind to a different but perhaps overlapping site. We then hypothesize that a hybrid peptide based on these two motifs may be more potent as inhibitors for $p60^{c\text{-}src}$ PTK. To test this hypothesis, we synthesized several branched chimeric peptides by combining portions of these two peptides together. One of these chimeric peptides, YI(2'-Nal)GK(N^{ϵ}-HHW)FK, has an IC_{50} value of 0.6 μM, significantly more potent than its linear counterparts (35).

Discussion

The "one-bead one-compound" combinatorial library method is an invaluable tool for basic research and drug discovery. This report highlights some of the assay methods available for this type of combinatorial library, and their applications to various signal transduction research. Since each member of the "one-bead one-compound" library is spatially separated but analyzed concurrently, it is not uncommon to discover multiple distinct motifs for a specific target. This parallel approach is very different from the combinatorial library methods that require deconvolution (3,10,11,12,13), or the affinity column selection approach by which only one predominant motif can be identified (18,19).

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